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Effects of two copper compounds on *Microcystis aeruginosa* cell density, membrane integrity, and microcystin release

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ABSTRACT

Microcystin release following *Microcystis aeruginosa* cell lysis after copper-based algaecide treatment is often cited as a concern leading to restricted use of algaecide in restoration of natural water resources. To examine this concern, bench-scale experiments were conducted to study responses of *M. aeruginosa* to 8-day copper exposures as copper sulfate and copper–ethanolamine (Cu–EA). *M. aeruginosa* UTEX 2385 was cultured in BG11 medium to cell density of 10^6 cells/mL with total and extracellular microcystin of 93 and 53 μg/L, respectively. Exposures of copper concentration ranged from 40 to 1000 μg Cu/L. Cell membrane integrity was indicated by erythrosine B. In the end of experiment, total microcystin and cell density in untreated control (313 μg/L and 10^7 cells/mL) was 3.3 and 10 times greater than pretreatment value, respectively. Minimum amount of copper required to reduce *M. aeruginosa* population within 8 days was 160 μg Cu/L as copper sulfate and 80 μg Cu/L as Cu–EA, where total and extracellular microcystin concentrations (47 and 44 μg/L for copper sulfate; 56 and 44 μg/L for Cu–EA) were degraded with degradation rate coefficient 0.1 day^{-1} and were less than pretreatment values. Given a copper concentration at 80 μg Cu/L as Cu–EA, *M. aeruginosa* cells were intact and less microcystin were released compared to treatments at 160–1000 μg Cu/L, where lysed cells and relatively greater microcystin release were observed. Based on the laboratory results, a minimum amount of copper required for reducing *M. aeruginosa* population could decrease total microcystin concentration and not compromise cells and minimize microcystin release.

1. Introduction

Microcystis aeruginosa commonly occurs in surface freshwaters and often produces microcystins, which are potent hepatotoxins and tumor promoters that can cause death in aquatic and terrestrial animals, as well as create problems for public drinking waters (Landsberg, 2002; Ouahid et al., 2005; Dittmann and Wiegand, 2006; Svrcek and Smith, 2004; Otten and Paerl, 2015). For most of a cell's life, microcystins are normally confined within cells of *M. aeruginosa* and enter the surrounding water after cell membrane lysis and death (Watanabe et al., 1992). Currently, more than 80 structural variants of microcystin have been identified (Westrick et al., 2010) and a provisional drinking water guideline of 1 μg/L (as total microcystin-LR, L: leucine, R: arginine) has been adopted by the World Health Organization (World Health Organization, 2011). Cell densities of *M. aeruginosa* in blooms have been observed as high as 10^9 cells/mL, and concentrations of total microcystin reported were generally density-dependent (Chorus et al., 2000; Zohary and Pais-Madeira, 1990). After blooms of *M. aeruginosa* are initiated, total microcystin concentrations would likely increase along with cell densities, if no action was taken to mitigate potential risks (Lehman et al., 2008). When utilization of water resources for human purposes such as drinking or recreation is threatened by *M. aeruginosa*, algaecide applications are often the preferred option for managing this noxious alga to rapidly restore the use of critical water supplies (Schrader and Dennis, 2005; Hoko and Makado, 2011).

Although algaecides and microbial biocides have been used for decades to control growth of noxious algae, some misconceptions regarding the consequences of their use persist. For example, public concerns often arise regarding perceived undesirable consequences of using copper-based algaecides for control of *M. aeruginosa* and some of these perceptions emerge from studies suggesting that loss of *M. aeruginosa* cell membrane integrity after applications of copper-based algaecides could result in release of intracellular microcystins into surrounding waters (e.g., Kenefick et al., 1993; Jones and Orr, 1994; Touchette et al., 2008; Polyak et al., 2013; Zhou et al., 2013; Fan et al., 2013). Using copper sulfate, Kenefick et al. (1993) studied release of microcystin from *M. aeruginosa* after treatment in the laboratory with chemical

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concentrations higher than commonly used in the treatment of surface waters due to exceptional algal biomass in their study and concluded that the high treatment (0.64 mg Cu as $CuSO_4$/L) could lyse cells and release microcystin. Jones and Orr (1994) measured microcystin release from a bloom of *M. aeruginosa* in a recreational lake treated with an unspecified concentration of chelated copper algaecide that was spot sprayed. They concluded that microcystin could be released from cells post-treatment and care should be taken in treating potable water supplies. Touchette et al. (2008) studied microcystin release from *Anabaena* and *Microcystis* in microcosm experiments treated with copper sulfate and urged caution when applying chemical treatments based on the increase in soluble toxin (but not total microcystin concentration). In a laboratory study by Zhou et al. (2013) employing *M. aeruginosa* at densities of 4×10^6 cells/mL, both the loss of cell integrity (using K^+ leakage as an indicator) as well as incremental increases in extracellular microcystin concentrations were correlated with copper sulfate concentrations applied (0.5 and 2.5 μM $CuSO_4$). Daly et al. (2007) and Fan et al. (2013) found that chlorination treatments (7–29 mg min/L) of *M. aeruginosa* also caused intact cell membranes to lyse and release microcystins into solution and the chlorine concentration required to achieve 100% lysis increased with increasing *M. aeruginosa* cell densities. Importantly, these studies do not incorporate untreated controls and therefore do not represent the consequences of not treating a developing bloom that is producing toxins. These studies note, however, that production of toxins is generally related to cell density and the toxins are released to the external environment upon cell lysis. Clearly, the potential risks due to toxin production and potential exposure would increase with time if an intervention to mitigate risks is not implemented.

Accurate risk assessment of applications of copper-based algaecides for control of *M. aeruginosa* blooms relative to toxin production and release requires quantitative knowledge of target algal cell densities and the algaecide concentrations applied. According to the WHO provisional drinking water guideline for microcystin (World Health Organization, 2011), total (summation of intracellular and extracellular) microcystin-LR in water resources is the metric for concern and risk assessment for water utilization. Even if intracellular microcystin is released after application of an algaecide, total microcystin concentrations as well as associated risks for water utilization may not increase since exposures would be limited after growth and reproduction of microcystin-producing cyanobacteria (e.g., *M. aeruginosa*) are inhibited. In drinking water treatment plants, *M. aeruginosa* cells and intracellular microcystin can usually be removed by sand filters (Hoeger et al., 2005). Since extracellular microcystins cannot be readily treated and removed from drinking water (Svrcek and Smith, 2004; Westrick et al., 2010; Zamyadi et al., 2012), risks associated with use of post-treatment water would be proportional to concentrations of extracellular microcystins in this specific case (Schmidt et al., 2002; Carmichael et al., 2001).

Persistence of microcystins in a water resource may also be a risk factor. Microcystins are chemically relatively stable, but they can be degraded in natural waters by indigenous bacteria and the decrease in microcystin concentrations with time usually follows first-order kinetics (Kenefick et al., 1993; Jones et al., 1994; Cousins et al., 1996; Bourne et al., 2006; Ishii et al., 2004). Factors affecting biodegradation rates of microcystins include the type of bacteria and environmental conditions such as temperature, nutrients, and pre-exposure of bacteria to microcystins (Ishii et al., 2004; Park et al., 2001; Surono et al., 2008; Rapala et al., 1994; Ho et al., 2007). Algaecide applications could also influence the persistence of microcystins in water resources. Copper is an active ingredient of many algaecides and bactericides. If copper-based algaecides are used to mitigate risks of toxin-producing *M. aeruginosa* in aquatic systems, indigenous bacterial populations as well as biodegradation rates of microcystins could be altered. Therefore, data regarding alteration of biodegradation rates of microcystins after applications of copper-based algaecides are also important for risk assessment.

Since exposures of copper-based algaecides cause different responses by target algal species (Murray-Gulde et al., 2002; Rodgers et al., 2010; Bishop and Rodgers, 2012; Calomeni et al., 2014), laboratory predictions of responses of *M. aeruginosa* to exposures of copper-based algaecides can provide important information regarding the consequences of using copper-based algaecides for reliable and substantial reduction of *M. aeruginosa* blooms. Additionally, measurements of extracellular microcystin concentrations can indicate the post-treatment release of this endotoxin, and measurements of total microcystin concentrations are used to make risk-based decisions regarding the utilization of water resources.

The specific objectives of this study were: (1) to measure responses of microcystin-producing *M. aeruginosa* UTEX 2385 cultured in BG11 medium to 8-day exposures of non-chelated copper compound (copper sulfate) and chelated copper compound (copper-ethanolamine) in terms of cell density and total and extracellular microcystin concentrations; (2) to determine cell membrane integrity of *M. aeruginosa* and released microcystin; and (3) to determine microcystin concentration degradation rate and half-live after exposures to two copper compounds.

2. Materials and methods

2.1. Algal culture

To study effects of copper-based algaecides on *M. aeruginosa* and microcystin release in the laboratory, it is important to conduct experiments using a strain of *M. aeruginosa* reliably producing microcystin. In laboratory studies using synthetic culture medium, *M. aeruginosa* UTEX 2385 is a microcystin-producer that induced DNA fragmentation in hamster kidney cells and mouse embryo fibroblasts primary cells (Rao et al., 1998; Makarewicz et al., 2009; Asselman et al., 2012). Therefore, in the present study *M. aeruginosa* UTEX 2385 [University of Texas at Austin, Austin, TX] was cultured in BG11 medium (Stanier et al., 1971) and maintained at a temperature of 24 ± 2 °C and a 12:12-h light–dark photoperiod illuminated by cool white fluorescent lighting at an intensity of 2100 lux. The initial cell density used for this study was 1.15×10^6 cells/mL in the exponential growth phase. At the initiation of the experiment, water characteristics of the algal growth solution (BG11 medium) were as follows: temperature $= 24 \pm 2$ °C; dissolved oxygen = 8.7–9.5 mg/L; pH = 7.8–8.0; conductivity = 1710–1730 μS/cm; alkalinity = 40 mg/L as $CaCO_3$; hardness = 40 mg/L as $CaCO_3$. In this study, post-treatment microcystin degradation was evaluated in a unialgal and non-axenic culture of *M. aeruginosa*. Given the pH, temperature, and fluorescent light intensity of this study, biodegradation is assumed to be the main degradation route (Lawton and Robertson, 1999; Tsuji et al., 1994) resulting in decline in post-treatment microcystin concentrations.

2.2. Preparation of copper compounds

Two copper compounds were used in this study: a copper salt (copper sulfate pentahydrate) (Sigma Chemical Co., St. Louis, MO, USA) and an ethanolamine-chelated copper compound (Cutrine®-Plus) (Lonza Inc., Germantown, WI, USA). The characteristics of copper sulfate and copper-ethanolamine (Cu-EA) are showed in Table 1. A stock solution (1000 mg Cu/L) was prepared